Corso di Dottorato di Ricerca in Scienze della Vita e dell'Ambiente

Ciclo XXXIX

Mediterranean loggerhead sea turtle as potential sentinel species for anthropogenic threats: biomarker identification and protocol development to evaluate effects on reproduction and embryo development

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Introduction and Aim:

The Mediterranean Sea is increasingly subjected to anthropogenic pressures that threaten marine biodiversity and ecosystem health. Among its vulnerable species, the loggerhead Sea Turtle (*Caretta caretta),* the most widespread sea turtle in the region, has been recognized as a key sentinel species for monitoring environmental contamination. It is currently designated as the official bioindicator for Descriptor 10 "Marine Litter" under the Marine Strategy Framework Directive. Throughout all life stages, from embryos to adults, Loggerheads are exposed to a complex mixture of pollutants, including microplastics (MPs), heavy metals (HMs), persistent organic pollutants (POPs), and phthalates. These substances can bioaccumulate and interfere with crucial physiological processes, potentially compromising individual health, development, and reproductive success. Alarmingly, the presence of such contaminants has also been detected in embryonic tissues, suggesting maternal transfer as an early exposure route. In this context, this study aims to identify selected biomarkers to detect the sub-lethal effects of these threats on *C. caretta* across different developmental stages, geographic areas, and temporal scales within the Mediterranean basin. By integrating morphological, physiological, toxicological and molecular approaches, the final goal is to develop standardized sampling and analytical protocols to support future conservation strategies and long-term monitoring programs tailored to this sentinel species.

Material and Methods and Preliminary Results:

Samples collection

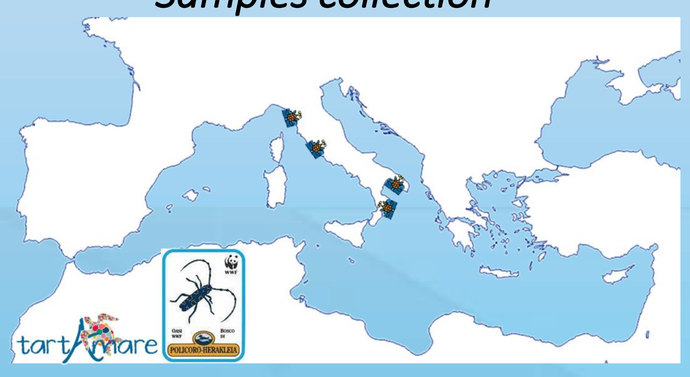
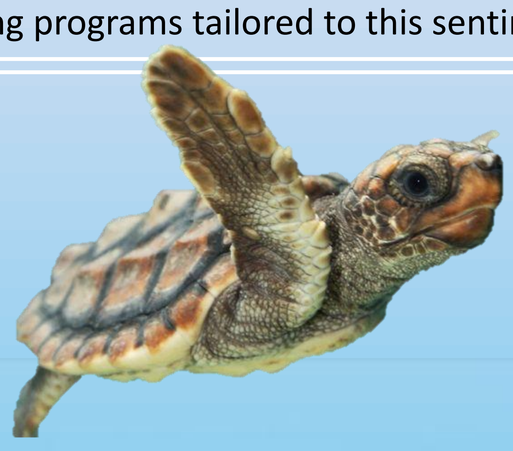
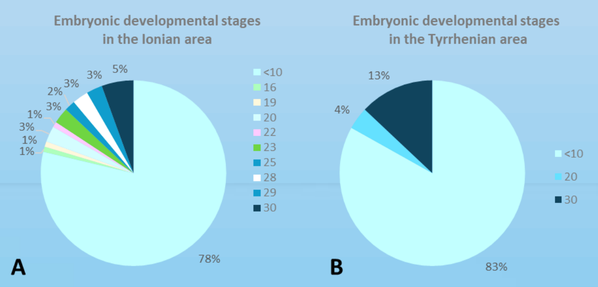


Figure 1.
Sampling sites of unhatched sea turtle embryos from the Ionian and Tyrrhenian Seas within the Mediterranean region in collaboration with WWF Policoro Herakleia and tartAmare rescue center.



Embryonic developmental stages



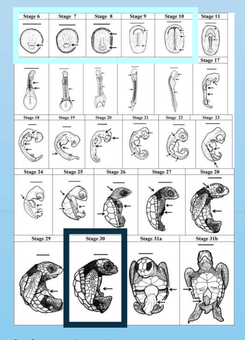


Figure 2.
Developmental stages of sampled eggs from the two study areas, Ionian Sea (A) and Tyrrhenian Sea (B). In both regions, most of the embryos had arrested development at early stages (<10) (light blue box) (78% in the Ionian Sea and 83% in the Tyrrhenian Sea), while only a small proportion showed developmental arrest at stages close to hatching (30) (dark blue box) (5% in the Ionian Sea and 13% in the Tyrrhenian Sea).

30 developmental stage

Biometric measurements

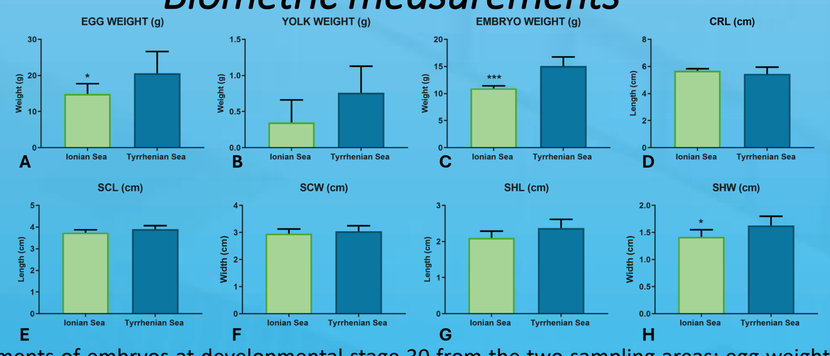


Figure 3.
Biometric measurements of embryos at developmental stage 30 from the two sampling areas: egg weight (A), yolk weight (B), embryo weight (C), crown-to-rump length (CRL) (D), straight carapace length (SCL) (E), width (SCW) (F), straight head length (SHL) (G), and width (SHW) (H). Ionian embryos generally showed lower values than Tyrrhenian ones, with significant differences in egg weight (p = 0.0208), embryo weight (p = 0.0008), and SHW (p = 0.0451).

Stress Assessment

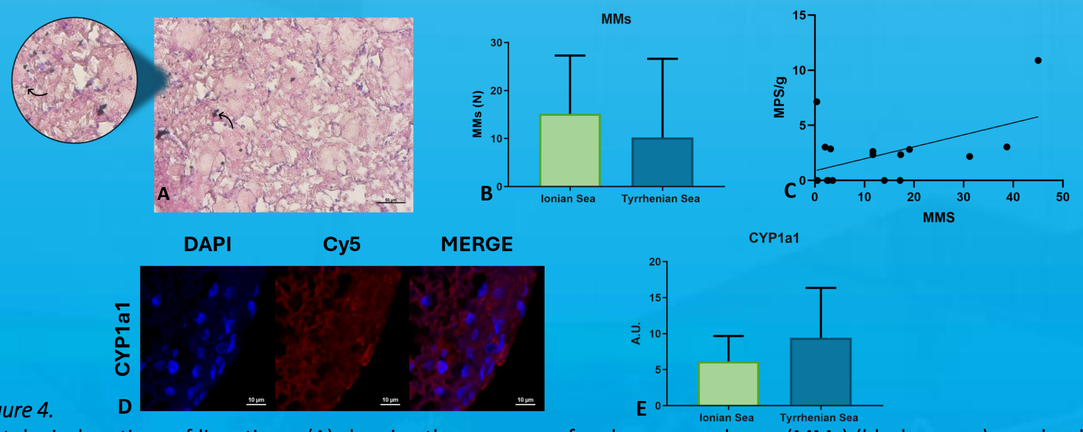


Figure 4.
Histological sections of liver tissue (A) showing the presence of melanomacrophages (MMs) (black arrows), used as biomarkers of environmental stress. Statistical analysis revealed no significant differences in the number of MMs between unhatched embryos at developmental stage 30 from the two sampling areas (B), but a significant positive correlation was observed between hepatic MMS and number of microplastic (C) (p = 0.0349; Pearson's r = 0.5137). Panel (D) shows immunofluorescence staining of CYP1A1 expression, with no significant differences detected between the two areas (E).

Pollutant assessment

		1		2		3		4		5		6		7	
		Y	L	Y	L	Y	L	Y	L	Y	L	Y	L	Y	L
LOW PAH	Naphthalene			4,89		<LOQ			<LOQ				<LOQ	<LOQ	
	Acenaphthene			11,97										<LOQ	
	2-Bromonaphthalene			0,97										<LOQ	<LOQ
	Acenaphthylene	<LOQ			2,34	<LOQ		2,29	9,91	10,11	1,76	0,93		3,80	7,55
	Fluorene					0,77		3,42		3,22	5,67				
	Phenanthrene					1,39		1,06					<LOQ		
	Anthracene	<LOQ			1,71				8,38			3,99		<LOQ	<LOQ
HIGH PAH	Fluoranthene							7,36	23,71			7,36			
	Pyrene				<LOQ	<LOQ			0,99	0,81					
	Benzo (a) anthracene							23,20	2,40	<LOQ					<LOQ
	Chrysene											1,00			
	Benzo (b) fluoranthene					<LOQ				<LOQ	<LOQ				
	Benzo (a) pyrene						2,70			<LOQ			<LOQ		
	Indeno (1, 2, 3) Pyrene												<LOQ		
	Dibenz (a, h) anthracene														
	Benzo (g, h, i) perylene								1,49		<LOQ				
	ΣLOW PAH			17,83	4,04	2,17		6,77	18,29	13,33	7,42	4,91		3,80	
	ΣHIGH PAH						2,70	30,56	28,58	0,81		8,36			
	TOTAL PAH			17,83	4,04	2,17	2,70	37,33	46,87	14,14	7,42	13,28		3,80	

Figure 5.
Quantitative analysis of polycyclic aromatic hydrocarbons (PAHs) was conducted using high-performance liquid chromatography (HPLC) in collaboration with UNICAM. PAH concentrations (ng/g) in yolk (Y) and liver (L) of seven embryos (1-7) at developmental stage 30 were measured. Red boxes indicate compounds detected in both matrices, suggesting potential maternal transfer.

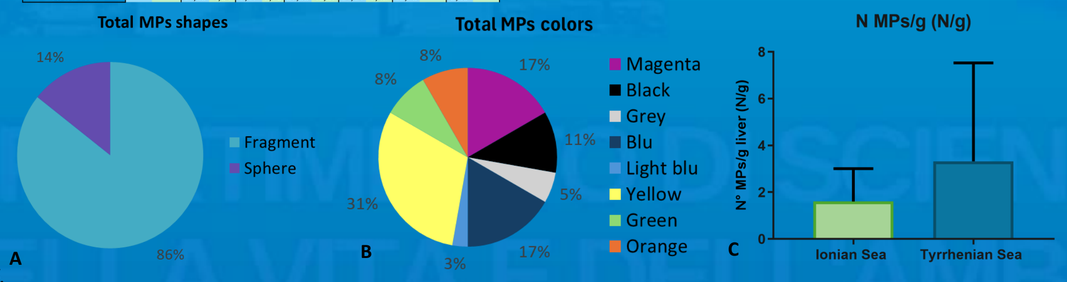
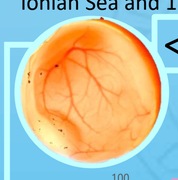


Figure 6.
Microplastic analysis in liver tissue of embryos at developmental stage 30 was conducted using Raman spectroscopy. Fragments (86%) and yellow particles (31%) were the most represented shapes (A) and colors (B), respectively. Statistical analysis (C) revealed no significant differences between the Ionian and Tyrrhenian sampling sites.

<10 developmental stages



Yolk metabolism assessment

Figure 7.
Graphical representation of protein pattern (A) and expression levels of cathepsins B, D, and L (B) in yolk samples from embryos at developmental stages <10, collected in the Ionian region. Analyses were performed using SDS-PAGE (A) and Western blotting (B). Results are compared across different sampling years to evaluate interannual variation.

Pollutant assessment

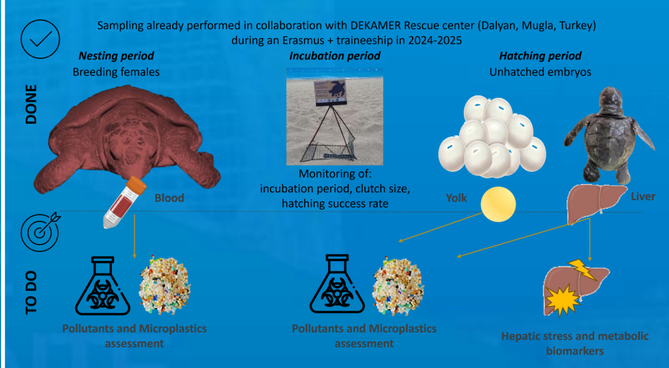
		Yolk (ng/g)				
		2015	2017	2019	2021	2022
LOW PAH	Naphthalene	8,7	1,6	6,8	1,4	5,1
	Acenaphthene	1,9		1,5	8,2	
	2-Bromonaphthalene	1,5	1	5,5		
	Acenaphthylene		1,9	1,5	4,8	1,8
	Fluorene	5,1	8,5	10,6		8,7
	Phenanthrene	6,1	16		4,4	7,4
	Anthracene	6,1	1,5	17,1		3
HIGH PAH	Fluoranthene		1,3	7,2		2,5
	Pyrene	1,2	2,9	1,7		0,8
	Benzo (a) anthracene	0,6	5			
	Chrysene	2,4	3			1,2
	Benzo (b) fluoranthene			0,8		
	Benzo (a) pyrene					
	Indeno (1, 2, 3) Pyrene			2,2		
	Dibenz (a, h) anthracene					
	Benzo (g, h, i) perylene					

Figure 8.
Quantitative analysis of polycyclic aromatic hydrocarbons (PAHs) conducted using HPLC. The table reports the maximum concentrations (ng/g) of PAHs detected in the yolk of embryos at developmental stages <10, collected from the Ionian region. Results are compared across different sampling years to assess temporal variation.

Discussion and Conclusions:

By comparing two different sampling areas in the Mediterranean Sea (Ionian and Tyrrhenian), significant differences were observed in the morphological and physiological development of *C. caretta* embryos. In particular, embryos from the Ionian area exhibited earlier developmental arrest and reduced biometric growth compared to those from the Tyrrhenian area. These findings highlight the need for further studies on contaminant effects during development and for the identification of specific biomarkers to support standardized monitoring and conservation efforts.

Future perspectives:



Future research will aim to confirm the maternal transfer of contaminants and to identify reliable biomarkers through the analysis of blood samples collected at DEKAMER (Turkey).

The ultimate objective is to develop standardized monitoring protocols for assessing embryonic development in Mediterranean sea turtles.